PMA-qPCR for Quantitative Differentiation of Live Human-associated *Bacteroides*

1. SCOPE AND APPLICATION

1.1. This method describes a quantitative polymerase chain reaction (qPCR) procedure targeting the 16S rRNA gene of human associated *Bacteroides* with a propidium monoazide (PMA) modification. This method is used for quantitative differentiation of live cells in water with the LIMS Test ID PMA-HF183.

1.2. PMA is a photoreactive DNA binding dye. The dye intercalates into double stranded DNA (dsDNA) upon exposure to intense visible light, resulting in chemically modified DNA that cannot be amplified with PCR. The dye cannot penetrate the cell membrane and will modify only exposed DNA from dead cells while leaving DNA from viable cells unmodified.

2. SUMMARY OF METHOD

2.1. Bacteria present in a water sample are collected by filtration. PMA is added to the filter and incubated in the dark. The sample is then exposed to high intensity light for photolysis to occur, resulting in the dye covalently reacting with exposed dsDNA from dead cells. DNA is extracted from the filter using a bead beating method with a DNA extraction kit. The extract is analyzed with qPCR.

2.2. Interferences: Particulate matter present in the environmental matrix can potentially limit the dye from accessing the DNA molecules. Concentrating solids or other inhibitors during filtration might interfere with the covalent bonding of PMA to DNA. Some substances, including humic and tannic acids, can act as inhibitors and reduce efficiency by either binding to the DNA polymerase or interfering with the annealing and extension cycles. Sample purification or dilution can help alleviate the effects of inhibitors.

3. EQUIPMENT AND SUPPLIES

3.1 Gloves, latex or nitriles

3.2. 50mL centrifuge tubes

3.3. Tube racks

3.4. Forceps

3.5. Microcentrifuge tubes, 1.5 and 2.0 mL

3.6. Polycarbonate membrane filter, 47-mm diameter, 0.45 µm pore size

3.7. Glass Fiber Filter, 47 mm diameter, AP20

3.8. Pipette pump with sterile disposable 50 mL tips

3.9. Micropipettes with sterile PCR grade filter tips

3.10. Magnetic Funnels with 47 mm bases, 500 mL

3.11. Vacuum Filtration Manifold

3.12. Petri dishes, 47 mm, sterilized, with rimmed lid

3.13. Autoclave (S/N 031761885)

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3.14. Fisher Mini Vortexer

3.15. Fisher Minicentrifuge

3.16. Centrifuge

3.17. Blue LED light manifold

3.18. Qiagen PowerLyzer Power Soil DNA isolation kit

3.19. Thermo Refrigerator #2 (S/N 0121179201121219), maintained at 4ºC ± 2ºC, Room B246B

3.20. Thermo Freezer #1 (S/N 0121174101121218), maintained at -20ºC ± 10ºC, Room B246A

3.21. Thermo Ultra Low Freezer (S/N 0132376801130826), maintained at -80ºC ± 10ºC, Room B246

4. REAGENTS AND STANDARDS

4.1 Dimethyl sulfoxide (DMSO)

4.1.1 DMSO is diluted to 20% DMSO (v/v) before use. Add 800 µL of PCR grade water to a 2 mL microcentrifuge tube. Add 200 µL of DMSO. Mix well by vortexing. Store 20% DMSO at room temperature in Room B246A on shelf above printer.

4.1.2 Record purchased DMSO and the working stock in LIMS Standard Preparation Tracker Module (**Laboratory/Technical/Standard Preparation Tracker**). Choose BIO-PCR from the "Group" drop down list.

4.1.2.1 For DMSO Purchased Reagent: Select Purchased/New Reagent from the menu. If a similar reagent record exists then select Purchased/Duplicate Reagent/ Material from the menu and choose the reagent to duplicate from the list.

4.1.2.1.1 Identity: = Dimethyl Sulfoxide (DMSO); Description: = Solvent used to dissolve PMA dye; Entered by: = Analyst Name; Type: = Solvent; Grade: = A.C.S. Grade; Purity: = Blank; Received Date: = Date of receipt; Expires: = Date of expiration if on the bottle; Has certificate of analysis: = Yes; Manufacturer: = MP Biomedicals; Lot # = from the bottle; Storage Location: = B212; Additional Information: = Stored in desiccator; Storage Temperature: = Room temperature; Container Size: = 100; Units: = mL.

4.1.2.2 For DMSO Working Stock: Select Prepared/New Reagent from the menu. If a similar reagent record exists then select Prepared/Duplicate Reagent from the menu and choose the reagent to duplicate from the list.

4.1.2.2.1 Identity: = 20% Dimethyl Sulfoxide; Type: = Working Reagent; Holding time: = 90 days; Entered by: = Analyst Name; Prepared: Date/Time/By: = Date and Time of preparation/ Analyst name; Location: = B246A; Storage Temperature: = Room temperature; SOP: = PCR-1.1; Description: = Solvent for making PMA dye; Solvent used: = Water; Final volume: = 1000; Units: = µL;

4.1.2.2.2 Click Add. In the "Enter Serial #" pop up window, choose the purchased reagent from the list, or enter its serial number in the text box. Click OK. In the "Enter Amount & Units for.." pop up window, enter "200 as Vol/Wt added" and "Units in µL". Click OK.

4.2 PMA dye (stored in a cardboard box at 4ºC, Room B246B)

4.2.1 Reconstitute PMA to a working stock concentration of 20 mM.

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Note: PMA is a photoreactive dye and must be worked with in darkness. Turn off the lights of the room and line the glass door with either black trash bags or aluminum foil.

4.2.2 Remove the PMA dye vial from 4ºC. Centrifuge to collect the powder dye at the bottom of the vial at ~7000 rpm for 30 sec.

4.2.3 Add 98 µL of 20% DMSO to the PMA dye vial to make a 20 mM stock.

4.2.4 Mix well by vortexing for 10 minutes at speed 6. Centrifuge briefly to coalesce drops.

4.2.5 Aliquot 10 µL of PMA in 500 µL microcentrifuge tubes.

4.2.6 Record purchased PMA and the working stock in LIMS Standard Preparation Tracker Module (**Laboratory/Technical/Standard Preparation Tracker**). Choose BIO-PCR from the "Group" drop down list.

4.1.2.1 For PMA Purchased Reagent: Select **Purchased/New Reagent** from the menu. If a similar reagent record exists, then select **Purchased/Duplicate Reagent/Material** from the menu and choose the reagent to duplicate from the list.

4.1.2.1.1 Identity: = Propidium Monoazide (PMA); Description: = photoreactive DNA binding dye; Entered by: = Analyst Name; Type: = Powder; Grade: = HPLC Grade; Purity: = Blank; Received Date: = Date of receipt; Expires: = Date of expiration if on the bottle; Has certificate of analysis: = Yes; Manufacturer: = Biotium; Lot # = from the bottle; Storage Location: = 246B; Additional Information: = None; Storage Temperature: = Refrigeration at 4degC; Container Size: = 1; Units: = mg.

4.1.2.2 For PMA Working Stock: Select **Prepared/New Reagent** from the menu. If a similar reagent record exists, then select **Prepared/Duplicate Reagent** from the menu and choose the reagent to duplicate from the list.

4.1.2.2.1 Identity: = 20mM PMA DYE; Type: = Stock Reagent; Holding time: = 365 days; Entered by: = Analyst Name; Prepared: Date/Time/By: = Date and Time of preparation/ Analyst name; Location: = B246A; Storage Temperature: = Freezer at < -10degC; SOP: = PCR-1.1; Description: = PMA DNA binding dye; Additional Information: = dissolved in 20% DMSO. Solvent used: = Water; Final volume: = 100; Units: = µL;

4.1.2.2.2 Click **Add**. In the "Enter Serial #" pop up window, choose the purchased reagent from the list, or enter its serial number in the text box. Click **OK**. Click **Add** again and choose 20% DMSO from the prepared list. In the "Enter Amount & Units for.." pop up window, enter "98 as Vol/Wt added" and "Units in µL". Click **OK**.

4.1.2.2.3 Click **OK** to save the entry and receive a serial number. Open the LabelMark6 software and create labels (see SOP PCR-5.0 for details). Print labels with the reagent name (20mM PMA), serial number, and expiration date and affix labels to the side of each tube. The top label should include the reagent name. Place labels on all aliquoted tubes before storage at -20°C.

4.3 Deionized Water

4.4 Molecular Biology grade water. Corning Molecular Biology Grade Water tested to USP Sterile Water (Cat. # MT46000CV).

4.5 Bleach solution, 10% (v/v)

4.6 Phosphate Buffered Saline (PBS) (Prepare per SOP PCR 3.0)

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4.7 TE buffer (# BP2473)

5. SAMPLE PREPARATION AND PMA TREATMENT

5.1. Filter 100-200 mL of water sample per SOP PCR 4.0. The volume filtered is determined by water turbidity and the number of filters needed for other analyses. Collect two filters per sample, one for PMA treatment to determine live cells and one PMA-untreated filter to represent total cells. For samples serving as duplicates, always filter 100 mL and collect four filters total, two for PMA treatment and two for untreated.

5.2 Using forceps, transfer the two collected filters to small petri dishes containing one drop of PBS. Petri dishes should be labeled with the sample ID# and PMA-treated or PMA-untreated. Make sure the filter is smooth and flat.

5.3 Working in the darkened room, make a 100 µmol working concentration of PMA, 300 µL per PMA-treated filter. For example, add 3 µL of 20 mM PMA in 597 µL of PBS in a microcentrifuge tube for treating 2 filter samples. Mix well and add 300 µL of PMA to each PMA-treated filter, covering it completely. The PMA-untreated filter will be incubated with 300 µL of PBS as a control.

5.4 Incubate the filters in the dark for 20 minutes with occasional gentle mixing to ensure entire filter remains in contact with the dye.

5.5 Expose filters to light by placing the petri dish under the LED lights in the manifold for 15 minutes. Remove the lids of the petri dishes before turning on the light.

5.6 Aseptically remove the filters from the petri dishes and place them in bead tubes from the Qiagen PowerLyzer Power Soil DNA isolation kit. Proceed with DNA extractions or freeze the filters at -80ºC until analysis.

5.7 Add samples and filtration information in Prep Worksheet Manager. PMA samples have the Test ID PMA-HF183 and the components HF183-PMA treated-qPCR and HF183-purified-untreated-qPCR. All samples are scheduled for both components and are added to worksheet type SOP-PCR-1.1.

5.7.1 Add up to 6 samples per worksheet. Duplicate each line and add _PUR to one of the lines for each sample. This will serve as the untreated control. The line with the sample ID alone represents the PMA treated filter.

5.7.2 Choose one sample to serve as the duplicate for the worksheet. This sample will have 4 lines total: _DUP_A_PUR, _DUP_A, _DUP_B_PUR, and _DUP_B.

5.7.3 After filtration, input the filtration date/time and volume. Include the LIMS ID for the PBS in Reagent 1 and the PMA dye in Reagent 2.

Note: Keep forceps in a small beaker of ethanol during the filtration and incubation process. Dip in the ethanol and shake off excess before grasping each filter. If forceps are accidentally dropped or placed on counter, replace with a new pair of sterile forceps from the UV sterilizer.

6. SAMPLE EXTRACTION

6.1 Extract total genomic DNA from filters using the Qiagen PowerLyzer Power Soil DNA isolation kit as per SOP 4.2.

6.2 Freeze extracts at -20º C until analysis.

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7. SAMPLE ANALYSIS

7.1 Refer to SOP PCR-1.0 for HF183 qPCR sample analysis. For PMA plates, the Target Name for the HF183 primer/probe in the StepOne/QuantStudio3 file is PMAHF. The spikes and standards are the same as in PCR-1.0, and the extraction control and purified (untreated control) samples have the purified nomenclature.

8. QUALITY CONTROL

8.1 Analyze a method blank (MB) for PMA treatment with the batch.

8.2 Analyze an extraction blank (EB) by placing a sterile filter in a bead tube and extracting it with the batch.

8.2 Analyze an extraction control (EC) by placing a sterile filter in a bead tube, spiking in 10^5 *B.dorei* DNA and extracting with the batch. This will be analyzed on the reaction plate to quantify recovery. Only one EC is required for PMA worksheets, as no other markers will be analyzed for these samples.

9. SAFETY/ HAZARDOUS WASTE MANAGEMENT

9.1 For sample filtration, extraction and analysis, wear safety glasses, gloves, and a clean disposable lab coat. If any substance falls on the gloves during processing, change into a new pair immediately to prevent any possible contamination of samples or controls.

9.2 Refer to the Laboratory Health and Safety Manual and Hazardous Waste Management.

9.3 Read MSDS information for reagent handling and disposal.

10. REFERENCES

10.1 DEP Laboratory Quality Manual

10.2 Health and Safety Plan

10.3 DEP SOP PCR-1.0, *Human-Associated Bacteroidales HF183 Marker in Water by Quantitative Polymerase Chain Reaction (qPCR) Assay*

10.4 DEP SOP PCR-3.0, *Preparation of Reagents for quantitative Polymerase Chain Reaction (qPCR)*

10.5 DEP SOP PCR-4.0, *Preparation of samples for qPCR*

10.6 DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen DNeasy PowerLyzer PowerSoil Kit*

10.7 DEP SOP PCR-5.0, *Sample Custody, Preparation Labels, and Worksheet Instructions for Molecular Biology*

10.8 DEP SOP PCR-5.1, *Data upload, Verification, and Sample Authorization for Molecular Biology*

10.9 Biotium/ PMA product information

Appendix of Changes

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